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mRNA Transcription Analyses of ROS Genes of Olea europaea L. In Vitro Cultures Treated with Different Boron Salts

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ABSTRACT: *Various factors such as biotic and abiotic stresses have effects on plant metabolism, development, and growth. Plants have many complex and extraordinary strategies to adapt, defend, avoid and tolerate all these stress conditions. In this study, the relative mRNA levels of antioxidant enzymes of olive, which is very difficult to reproduce under* ***in vitro*** *conditions, were assessed under oxidative stress conditions, after treatment with boron compounds. In this context, three different compounds of the element boron, which are known to affect the ascorbate-glutathione pathway, were added separately at two different concentrations to the nutrient medium of olive under in vitro conditions. As a result of the study, it was observed that the relative mRNA expression levels of antioxidant enzymes such as catalase, ascorbate peroxidase, and superoxide dismutase decreased only in the H_3BO_3 group among the experimental groups. An increase in the relative mRNA expression levels of antioxidant enzymes was observed in the $NaBO_2$ and $ZnBO_3$ groups compared to the control group. This situation was interpreted as due to an increase in salinity stress which thereby increased the oxidative stress of the applied $NaBO_2$ and $ZnBO_3$ groups. However, in the H_3BO_3 group, although the concentration was increased twofold, a decrease was observed in the relative mRNA expression levels of the antioxidant enzymes examined. This reveals that application concentration, as well as the compound used, is extremely important.*

Keywords: *Ascorbate peroxidase, boric acid, catalase, sodium metaborate, superoxide dismutase.*

Farklı Bor Tuzları ile Muamele Edilen Olea europaea L.'nin In Vitro Kültürlerinde ROS Genlerinin mRNA Transkripsiyon Analizleri

ÖZ*: Biyotik ve abiyotik stresler gibi çeşitli faktörler bitki metabolizması, gelişimi ve büyümesi üzerinde etkilidir. Bitkiler tüm bu stres koşullarına uyum sağlamak, savunmak, kaçınmak ve tolere etmek için birçok karmaşık ve olağanüstü stratejiye sahiptir. Bu çalışmada, in vitro koşullarda çoğaltılması oldukça zor olan zeytinin antioksidan enzimlerinin oksidatif stres koşullarında, bor bileşikleri sonrası göreceli mRNA seviyeleri incelenmiştir. Bu kapsamda, askorbat-glutatyon yolağını etkilediği bilinen bor elementinin üç farklı bileşiği, in vitro koşullarda zeytinin besin ortamına iki farklı konsantrasyonda ayrı ayrı ilave edildi. Çalışma sonucunda katalaz, askorbat peroksidaz ve süperoksit dismutaz gibi antioksidan enzimlerin rölatif mRNA ekspresyon seviyelerinin deney grupları arasında sadece H3BO3 grubunda azaldığı gözlendi. Kontrol grubuna kıyasla NaBO2 ve ZnBO3 gruplarında antioksidan enzimlerin rölatif mRNA ekspresyon seviyelerinde artış gözlenmiştir. Bu durum, uygulanan NaBO2 ve ZnBO3 gruplarında tuzluluk stresinin ve dolayısıyla oksidatif stresin arttığı şeklinde yorumlanmıştır. Ancak H3BO3 grubunda konsantrasyon iki kat artmasına rağmen antioksidan enzimlerin göreceli mRNA ifade seviyelerinde düşüş gözlenmiştir.*

Anahtar Kelimeler: Askorbat peroksidaz, borik asit, katalaz, sodyum metaborat, süperoksit dismutaz.

INTRODUCTION

Olea europaea L. subsp. *europaea* var. *sylvestris* Brot., known as wild olive, belongs to the Oleaceae family, which includes plants with high economic and cultural values such as ash (*Fraxinus* spp.), lilac (*Syringa* spp.), and jasmine (*Jasminum* spp.) (Akhtar *et al.,* 1994; Besnard *et al.*, 2001; Belaj *et al.*, 2002). Although wild olive is native to temperate Asian regions, it was also taken to the Americas and Australia continents with the discovery of new regions in the world, and today it spreads naturally in these regions (Baldoni *et al.*, 2006; Kaya and Yılmaz-Gokdogan, 2016; Besnard *et al.*, 2018).

Micropropagation can be defined as producing new plants with the same or very similar genetic characteristics from a plant produced *in vitro* by using organs and structures such as shoots, roots, and stems, from which the plant can form new micro-shoots (Ozudogru *et al.*, 2011; Contento *et al.*, 2002; Espinosa-Leal *et al.*, 2018). Micropropagation techniques are applied to many plant species and used in important commercial sectors such as secondary metabolite production, agriculture and forestry, and especially in scientific studies (Ozden-Ciftci *et al.*, 2010; Guerriero *et al.*, 2018; Kaya *et al.*, 2018; Mehbub *et al.*, 2022).

Micropropagation in olives, on the other hand, has not yet taken the place of traditional production techniques (such as cutting, grafting) due to high costs. In addition to the low shoot proliferation rate in the micro-propagated olive plant, root formation is very difficult. Moreover, losses after *in vitro* transfer are extremely high. Despite all these difficulties, olive micropropagation can be preferred as an alternative production model for commercial and conservation purposes (Rugini, 1984; Lambardi *et al.*, 2012; Galatali *et al.*, 2021).

Artificial nutrient media are used for growing plants *in vitro* (Rugini, 1984), and contain all the macroelements and microelements that a plant may need, as well as a carbon source, plant growth regulators and some chemical compounds that affect plant growth (Souza *et al.*, 2017; Kıvrak-Kıran *et al.*, 2021). In this study, different compounds of the element boron, which is thought to be effective on membrane proteins and cell wall strength, were used. Boron is known to have a role in wall membrane stabilization by acting on metal chelates in the cell wall and cell membrane (Murashige and Skoog, 1962; Clarkson and Hanson, 1980; Galatali *et al.*, 2021b). At the same time, boron is required for phenolic substance production, lignin synthesis, meristematic development and RNA synthesis (Tanada, 1978). Apart from its several other roles, boron plays an effective role in the gravity response and in the regulation of phenolase enzyme activity by phytochromes (Nable *et al.*, 1997).

Oxidative stress emerges as a side effect of many biotic and/or abiotic stresses in plants as well as in all living things, by increasing reactive oxygen species (ROS) such as OH^-, O_2^-, R-OO, H_2O_2 and affecting cell function (Gill and Tuteja, 2010; Krumova and Cosa, 2016; Agar *et al.*, 2022). The amount of ROS accumulated by oxidative stress in plants can be reduced with non-enzymatic antioxidant substances such as ascorbic acid, α-tocopherol, β-carotene, and antioxidant enzyme systems such as catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) (Shigeoka *et al.*, 1980; Diaz-Vivancos *et al.*, 2015; Sharma *et al.*, 2019).

Higher plants, red algae, and protists all contain the enzyme APX, which functions in many cell compartments (Takeda *et al.*, 1998; Takeda *et al.*, 2000). APX plays an important role in the control of ROS levels (Caverzan *et al.*, 2012) by acting on the ascorbate-glutathione cycle as an electron donor. APX is extremely effective in catalyzing the conversion of H_2O_2 to O_2, and H_2O, and this is highly effective in the cell's tolerance to oxidative stress (Sharma and Dubey, 2007). CAT is also effective in scavenging H_2O_2 like APX, but is released from the peroxisome in the cell (Shi *et al.*, 2012). The increase in H_2O_2 in the cell can empty the ascorbate stores. This triggers CAT activity (Nordgren and Fransen, 2014). Electron flow between glutathione and ascorbate can be accomplished both chemically and by dehydroascorbate reductase (DHAR). In the absence of CAT in the cell, the increase in APX and DHAR occurs both at the protein and

transcriptional levels, confirming the effect of CAT on the ascorbate-glutathione cycle (Willekens *et al.*, 1997; Foyer and Mullineaux, 1998). Another antioxidant enzyme that is effective in oxidative stress tolerance is SOD. By participating in certain cell compartments like the chloroplast, mitochondria, and peroxisome, it catalyzes the formation of H_2O_2 from ROS, which are more hazardous than H_2O_2 (Vanderauwera *et al.*, 2005; Corpas *et al.*, 2008).

The main purpose of this study was to increase the success of *in vitro* production by increasing the oxidative stress tolerance of the olive plant, which is very difficult to grow under *in vitro* conditions because it is under oxidative stress. In this context, it was aimed to examine the antioxidant enzymes APX, CAT and SOD, which are considered as markers of oxidative stress, at the mRNA level after boron application by using compounds of the element boron, which are effective on plant growth and ascorbate glutathione pathway.

MATERIALS AND METHODS

Plant samples and *in vitro* culture establishment

The seeds of *O. europaea* were obtained from the seed collection of the wild-type olive population (Muğla Metropolitan Municipality Local Seed Center). The wild-type olive seeds were decontaminated via modifying the surface sterilization methods developed by Kaya *et al.* (2020) (70% ethanol for 10 minutes, 10% hydrogen peroxide for 10 minutes, 20% commercial bleach for 20 minutes and sterile distilled water for rinsing). After the hard testa of the sterilized wild-type olive seeds were partially or completely broken using sterile pliers, they were transferred to semi-solid Rugini Olive Medium (OM) (Rugini, 1984) enriched with 1 mg l^{-1} benzyl adenine (BA).

Boron salt treatments

The explants (derived from *in vitro*-germinated wild-type olive seeds after four-week incubation) cut from *O. europaea* were transferred to Magenta™ tissue culture vessels contained two different concentrations (25 or 50 mg l^{-1}) of three different boron compounds (boric acid, sodium metaborate, or zinc borate, and each medium combination was enriched with 1 mg l^{-1} BA and 25 mg l^{-1} Fe-EDHHA. Three explants were used for each treatment and each parameter was repeated at least three times. The olive micro-shoots were incubated for seventy-two hours under standard culture conditions (16/8-hour photoperiod, 25±2°C, 50µmol^{-1}m-2s^{-1} white cold fluorescent lighting).

Each boron compound treatment was evaluated separately. After the isolation of total RNA (Thermo Fisher Scientific #K801) of the explants incubated for seventy-two hours (in order to allow the cut shoots to repair themselves during this period and to ensure the necessary adaptation to the ambient conditions) in nutrient medium containing three different boron compounds at two different concentrations, cDNA synthesis (Thermo Fisher Scientific #K1622) was performed from each isolate separately.

qPCR analyses

Boron compound treatment (25 and 50 mg l^{-1} of boric acid, sodium metaborate, or zinc borate) samples and ascorbate peroxidase (APX) in the control group, transcriptional levels of catalase (CAT), Mn-superoxide dismutase (Mn-SOD), Cu/Zn-superoxide dismutase (Cu/Zn-SOD), and Fe-superoxide dismutase (Fe-SOD) gene expression were analyzed using qPCR. For internal control group, β-actin (ACT) was used. The original sample not subjected to any boron compound treatment was the reference, and the samples subjected to the boron compounds treatment were the target samples (Çiçek et al., 2023). Information about the primers used in the current work are given in Table 1.

RT-qPCR experiments were performed using Roche Light Cycler-96. All amplifications were achieved using Ampliqon RealQ Plus 2 × Master Mix Green without ROX™ (#A323402). Thermal cycling conditions include one cycle for 15 min (900 s) at 95°C (pre-denaturation), 40 cycles for 30 s at 95°C (denaturation), 40 cycles of 30 s bonding to the primer at appropriate temperature, 30 s extension at 72°C and 40 cycles of elongation at 37°C. This was followed by indefinite cooling at 4°C .

Table 1**.** Primers used in the current work.

Gene		Sequence	Reference	Size
Cu/Zn-SOD	Forward	5'-GGCTGTATGTCAACTGGACCTCATTTCA-3'	(Mercan *et al.,* 2022)	140 bp
	Reverse	5'-TGTCAACAATGTTGATAGCAGCGGTG-3'		
Fe-SOD	Forward	5'-AACAAGCAAATAGCCGGAACAGAACTAAC-3'	(Mercan *et al.,* 2022)	128 bp
	Reverse	5'-AGAAATCGTGATTCCAGACCTGAGCAG-3'		
Mn-SOD	Forward	5'-AGTCAAGTTGCAGAGTGCAATCAAGTTC-3'	(Mercan *et al.,* 2022)	144 bp
	Reverse	5'-CAAAGTGATTGTCAATAGCCCAACCTAAAG-3'		
APX	Forward	5'-ATGTCCTGAAGAGGGGAGGC-3'	(Mercan *et al.,* 2022)	157 bp
	Reverse	5'- GCTCCAGGTCCTTCTTTCGTAT-3'		
CAT	Forward	5'- ATATCTCATCTTACCTGCGCTG –3'	(Corpas *et al.*, 2006)	164 bp
	Reverse	5'- AGATCAAAGTTTCCCTCTCTGG-3'		
ACT	Forward	5'-GGACCTGTATGCCAACACTG-3'	(Corpas *et al.*, 2006)	172 bp
	Reverse	5'-TGATCTCCTTCTGCATCCTG-3'		

Statistical analyses

In this study, we used the ΔΔCT (Livak Method) (SWU, 2022) method, which is the most preferred technique for qPCR data analysis. Within the scope of the method, the relative difference between the genes of interest and the housekeeping gene was calculated according to Equation 1.

$$\Delta Ct = Ct\ (gene\ of\ interest) - Ct\ (housekeeping\ gene) \quad (1)$$

ΔΔCt values were calculated by subtracting the mean ΔCt of the control group from the ΔCt values of the experimental groups (Equation 2).

$$\Delta\Delta Ct = \Delta Ct\ (experimental\ group) - \Delta Ct\ (control\ group\ mean) \quad (2)$$

The experimental group/control ratio was calculated by averaging the $2^{-(\Delta\Delta Ct)}$ values. It is recommended to convert this ratio to a logarithm value (such as log10) of the final gene expression to perform parametric statistical tests with the t-test. However, in our study, we multiplied the obtained ratio by one hundred to see the percentage changes in the data. For the t-test, the product of $2^{-(\Delta\Delta Ct)}$ values by one hundred, the two tail test and assuming unequal variance were chosen. If the resulting p values were less than 0.05, we were able to accurately establish the statistical significance of the differences between means of the data regarding the two sample means (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

The effect of concentration of boron compounds on changes in relative mRNA expression levels of antioxidant enzymes

When the explants incubated for seventy hours in a medium containing three different boron compounds at different concentrations were compared with the control group, a very high increase in the relative transcription levels of the *APX* gene was observed in all experimental groups. In contrast, only a small increase of 39% was observed compared to the other groups for the 50 mg l^{-1} H_3BO_3 treatment (Figure 1).

After the boron compound treatment, a very high increase was monitored in the relative transcription levels of the catalase gene in all groups compared to the control group, while a 36% decrease was observed only in the 50 mg l^{-1} H_3BO_3 group (Figure 2).

When the relative transcription levels of Mn-SOD, and Cu/Zn-SOD were investigated (Figure 3 and Figure 4), a profile similar to the CAT enzyme was observed. A decrease of 32% was monitored in the 50 mg l^{-1} H_3BO_3 group of the Mn-SOD gene, and an increase of 33% in the 50 mg l^{-1} H_3BO_3 group of the Fe-SOD gene (Figure 5).

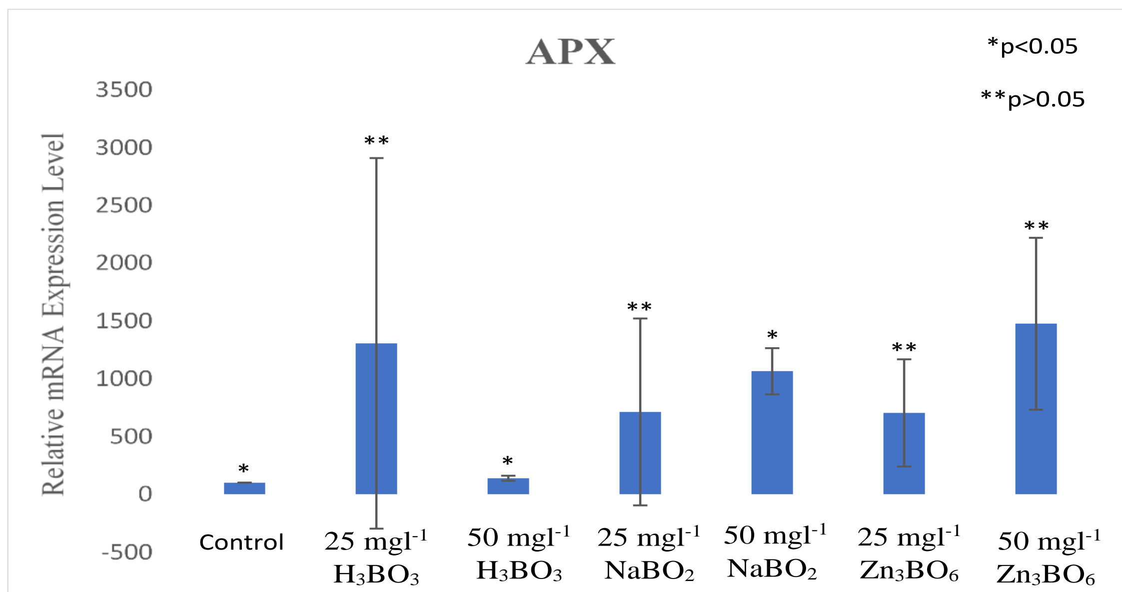

Figure 1. Relative transcription levels of the *APX* gene after boron compounds treatment.

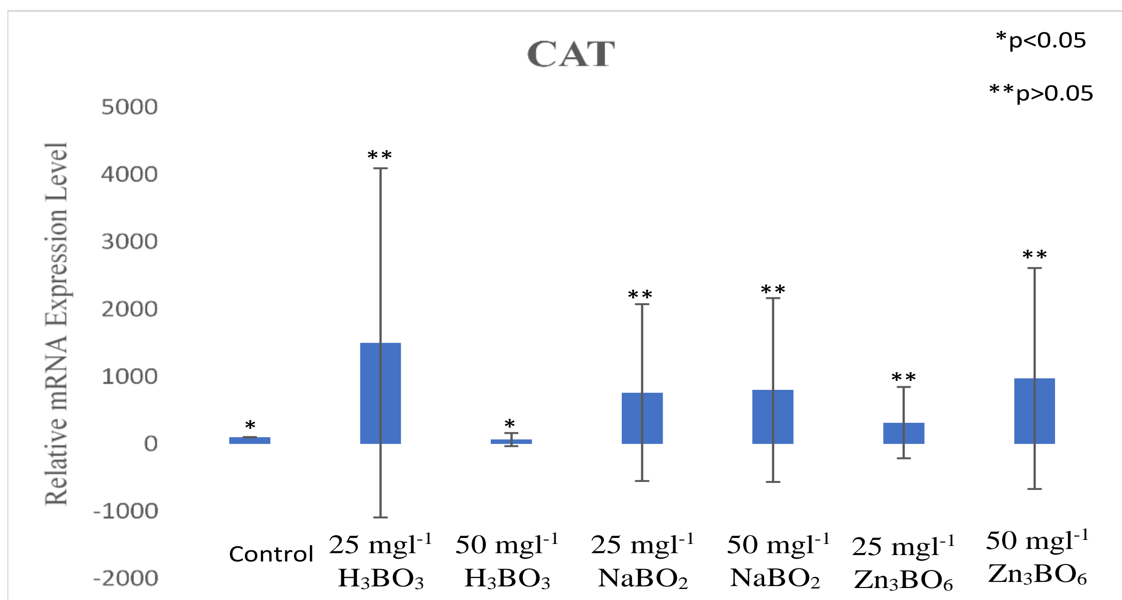

Figure 2. Relative transcription levels of the *CAT* gene after boron compounds treatment.

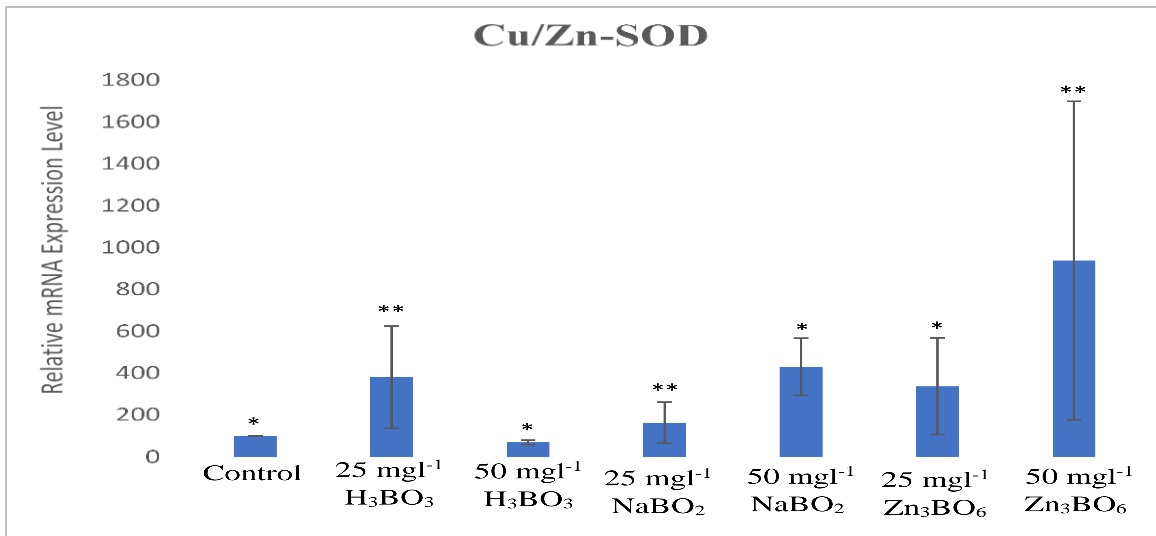

Figure 3. Relative transcription levels of *Cu/Zn-SOD* gene after treatment of boron compounds (*$p<0.05$; ** $p>0.05$).

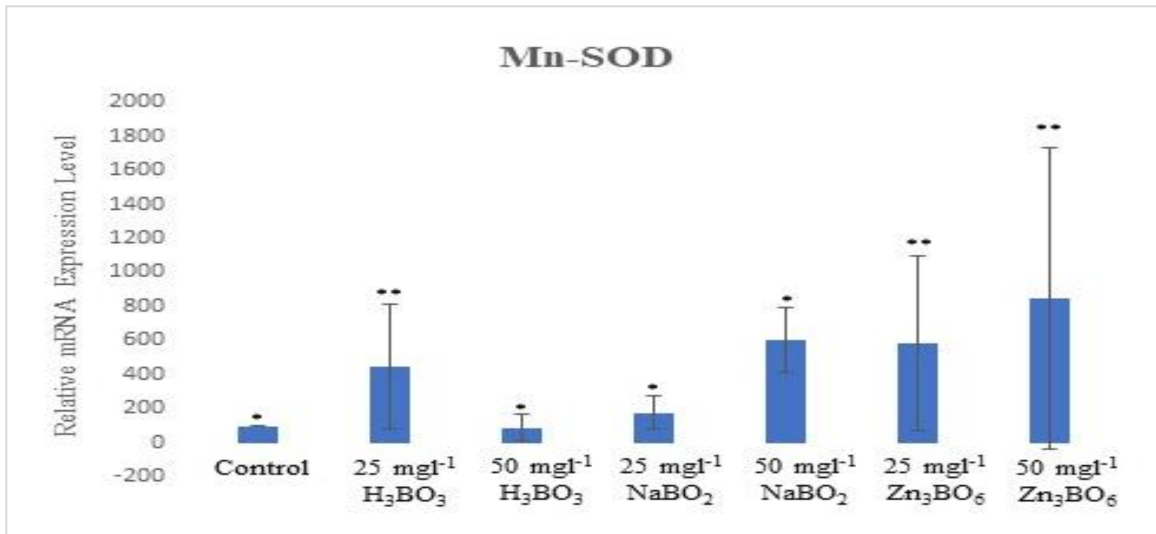

Figure 4. Relative mRNA expression levels of *Mn-SOD* gene after treatment of boron compounds (*$p<0.05$; ** $p>0.05$).

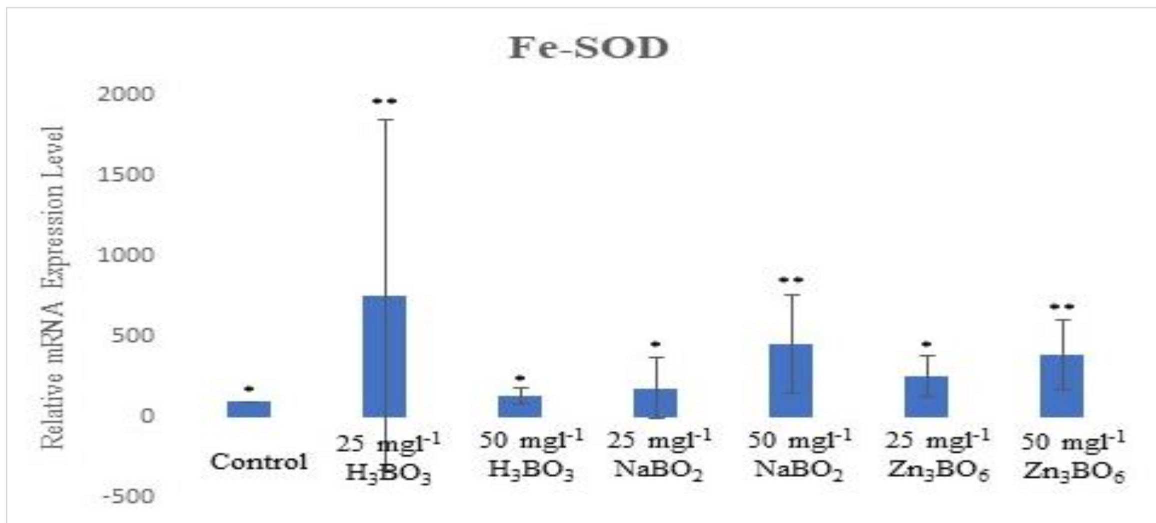

Figure 5. Relative transcription levels of *Fe-SOD* gene after treatment of boron compounds (*$p<0.05$; ** $p>0.05$).

Our current study findings are similar to the applications of boron and other salt compounds used previously. Mercan *et al.* (2022) obtained the best regeneration for *Liquidamber orientalis* from nutrient medium supplemented with 1 mg l^{-1} disodium octaborate, and in the genetic analysis studies, all of the micro-shoots obtained were genetically stable. At the same time, the highest shoot forming capacity was obtained from the nutrient medium supplemented with 5 mg l^{-1} sodium perborate. In addition, boron and boron salts gave effective results in protection against pathogens in plants. For example, it has been reported that boric acid and borax applications used in a study using different salts and fungicides against different fungi causing fruit rot in tomatoes significantly reduced the disease severity in tomatoes compared to the pathogen and inoculated control (Akhtar *et al.*, 1994). Shi *et al.* (2012) reported that potassium tetraborate against anthracnose disease in mango fruits decreased by 47% compared to untreated control fruits.

CONCLUSIONS

Since plants are living creatures that cannot move actively, they are affected by environmental stressors throughout their life cycle. Plants have developed physiological, morphological, biochemical and molecular defense systems to protect themselves from such stresses. It is imperative to possess adequate understanding of these systems in order to maintain plant life under dynamic climatic conditions, as well as to mitigate the effects of rising global population and hunger.

In this study, various treatments were carried out to reduce oxidative stress, which occurs as a side effect of all stress conditions. It was aimed to increase the development of the olive plant, in *in vitro* conditions which is difficult. In this context, boron, which is known to affect the ascorbate-glutathione pathway and required for plant growth, was examined for its effects on oxidative stress and relative mRNA expression via antioxidant enzymes. As a result of the data obtained, promising decreases were observed in the relative mRNA expression levels of all genes examined after 50 mg l^{-1} H_3BO_3 treatment of the olive plant exposed to oxidative stress *in vitro*. However, in $NaBO_2$ and Zn_3O_6 treatments, oxidative stress was expected to decrease or remain constant, but an increase was observed. This shows that oxidative stress in the plant increases. It can be deduced that the increase in oxidative stress increases the salinity stress due to the fact that some of the compounds used are salts, and accordingly, it resulted in an increase in oxidative stress. However, the fact that there was an increase in 25 mg l^{-1} H_3BO_3 administration compared to the control suggests that the applied dose may also be effective. As a result, the effects of oxidative stress in olive plants grown *in vitro*, depending on the selected compounds and the applied doses, were shown at the relative mRNA expression level. Although there are statistically insignificant data in this study, it is meaningful and promising for the H_3BO_3 application of the study and will guide studies to be done in the future. More boron compounds and studies that will be carried out by varying the dosage frequencies will provide more information about the effects of the element boron on the elimination of oxidative stress.

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